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# Macrocyclic trichothecene production and sporulation by a biological control strain of *Myrothecium verrucaria* is regulated by cultural conditions

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## Abstract

*Myrothecium verrucaria* is a pathogen of several invasive weed species, including kudzu, and is currently being evaluated for use as a bioherbicide. However, the fungus also produces macrocyclic trichothecene mycotoxins. The safety of this biological control agent during production and handling would be improved if an inoculum could be produced without concomitant accumulation of macrocyclic trichothecenes. Sporulation and trichothecene production by *M. verrucaria* was evaluated on standard potato dextrose agar (PDA) and a series of complex and defined media. Sporulation on PDA and on agar media with nitrogen as ammonium nitrate or potassium nitrate was more than ten-fold greater then sporulation on the medium with ammonium sulphate as the nitrogen source. Accumulation of macrocyclic trichothecenes was strongly affected by the media composition, with higher levels often associated with higher carbon content in the media. Overall, incubation in continuous darkness resulted in higher macrocyclic trichothecene concentrations. Results support the hypothesis that accumulation of macrocyclic trichothecenes by this fungus can be altered by manipulating carbon and nitrogen sources. Furthermore, the biosynthesis of these mycotoxins may be independent of sporulation, demonstrating that the bioherbicide can be readily produced on solid substrates while simultaneously yielding conidia that are less threatening to worker safety. A more detailed implementation of the concepts demonstrated in this study will facilitate the safe and economical production of this bioherbicide.

**Keywords**: bioherbicide, verrucarin A, trichothecenes, secondary metabolism

## 1. Introduction

*Myrothecium verrucaria* is a pathogen of several weedy plant species (Yang and Jong, 1995). Research with another strain of *M. verrucaria* (IMI 361690) confirmed the utility of this fungus as a potential bioherbicide against kudzu, morning glory, redvine and trumpet creeper (Boyette *et al*., 2006; Hoagland *et al*., 2007; Walker and Tiley, 1997). In addition to the high virulence of *M. verrucaria*, it is compatible with several commercially available herbicides (Boyette *et al*., 2008a; Weaver and Lyn, 2007). The association of the fungus with macrocyclic trichothecenes, however, raises safety concerns. Previous studies investigating the presence of trichothecenes in plant tissue after *M. verrucaria* application showed that these mycotoxins are undetectable (Abbas *et al*., 2001) or are present, briefly, at trace levels (Millhollon *et al*., 2003). If trichothecenes are not produced by *M. verrucaria*, *in planta*, then they only present a safety concern during bioherbicide production, formulation and application.

There are numerous published reports on the role of cultural conditions on fungal secondary metabolism. For example, nutritional factors and their interactions and effects on aflatoxin production in culture (Klich, 2007) and in field conditions (Scheidegger and Payne, 2003) have been reviewed. The relationship between aflatoxin biosynthesis and spore and sclerotia production has also been extensively studied (e.g. Hicks *et al*., 1997; Calvo *et al*., 2002; Chang *et al*., 2002). Observations relating cultural conditions and substrate type are available for fumonisin production (e.g. Hinojoa *et al*., 2006; Bluhm and Woloshuk, 2005; Etcheverry *et al*., 2002; Shim and Woloshuk, 1999) and other mycotoxins (Llorens *et al*., 2004; Hestbjerg *et al*., 2002) by *Fusarium* species. In contrast, only a single report is available describing the role of nutritional factors on roridin production by *M. roridum* (Reddy *et al*., 1998) and one observation for trichothecene production in a solid and two liquid media (Abbas *et al*., 2001). A biochemical pathway has been proposed and three genes involved in the biosynthesis of macrocyclic trichothecenes have been identified in *M. roridum* (Trapp *et al*., 1998; McCormick and Alexander, 2007), but environmental factors or exogenous signals regulating their expression have not been determined.

In the production of any bioherbicide, there are the competing goals of minimising production costs and incubation time while maximising yield. Because of the toxicological concerns with *M. verrucaria*, an additional goal is to identify conditions that allow abundant conidia formation, but also minimise the production of trichothecene mycotoxins. This research details the effect of carbon and nitrogen sources, culture age and illumination on conidia production and trichothecene levels.

## 2. Materials and methods

### Culture conditions

*M. verrucaria* (IMI 361390) was grown on potato dextrose agar (PDA) (Difco, Detroit, MI, USA) or a modified Vogel's medium (Vogel, 1956) (VM) consisting of: 16 g agar, 2.5 g sodium citrate (dihydrate), 5 g potassium phosphate monobasic (anhydrous), 200 mg magnesium sulphate (heptahydrate), 100 mg calcium chloride, 5 mg citric acid monohydrate, 5 mg zinc sulphate (heptahydrate), 1 mg ferrous ammonium sulphate (hexahydrate), 250 µg cupric sulphate (heptahydrate), 50 µg manganese sulphate (monohydrate), 50 µg borate (anhydrous), and 50 µg sodium molybdate (dihydrate)/l deionised water. Selected carbon and nitrogen sources (indicated in Table 1) were added to this defined medium. Incubations were in 8 ml of media in 60 mm diameter Petri dishes at 26 °C with a 12 hour light/12 hour dark cycle. For light exclusion incubations, Petri dishes were wrapped in aluminium foil and placed in an incubator without light. Conidia were collected in water after 3, 5, 9 or 15 days of incubation. Spore yield was determined by counting colony forming units after serial dilutions and plating on PDA. Elemental carbon and nitrogen content of complex media was measured using a Flash EA 112 elemental analyser (CE Elantech, Lakewood, NJ, USA) using glutamine as the carbon and nitrogen standard. Quantification was conducted in triplicate using a 100 µl injection volume.

**Table 1. Media composition.**

| Media | Description | Nitrogen[b] (g/l) | Carbon[b] (g/l) | C:N ratio |
|---|---|---|---|---|
| 1 | Potato Dextrose Agar | 0.92 | 9.97 | 10.9 |
| 2 | Vogel's medium[a]<br>Ammonium nitrate 2 g/l<br>Sucrose 15 g/l<br>Yeast Extract 2 g/l | 0.91 | 7.82 | 8.6 |
| 3 | Vogel's medium<br>Ammonium nitrate 2 g/l<br>Molasses 30 g/l | 0.82 | 12.87 | 15.7 |
| 4 | Vogel's medium<br>Ammonium nitrate 2 g/l<br>Molasses 15 g/l | 0.72 | 6.63 | 9.2 |
| 5 | Vogel's medium<br>Ammonium nitrate 2 g/l<br>Glucose 15 g/l | 0.7 | 6.61 | 9.4 |
| 6 | Vogel's medium<br>Ammonium nitrate 2 g/l<br>Sucrose 15 g/l | 0.7 | 6.92 | 9.9 |
| 7 | Vogel's medium<br>Ammonium nitrate 2 g/l<br>Sucrose 30 g/l | 0.7 | 13.23 | 18.9 |
| 8 | Vogel's medium<br>Ammonium sulphate 2.4 g/l<br>Sucrose 15 g/l | 0.51 | 6.92 | 13.6 |
| 9 | Vogel's medium<br>Potassium nitrate 2.4 g/l<br>Sucrose 15 g/l | 0.33 | 6.92 | 21.0 |

[a] Additional details of Vogel's medium is in the materials and methods.
[b] Carbon and nitrogen concentrations calculated for defined media and measured by elemental analysis for complex media.

### Trichothecene detection and quantification

Trichothecenes were extracted from replica cultures by transferring cultures (agar, mycelia and conidia) to conical centrifuge tubes and shaking with a 1:1 ratio (culture mass:ethanol volume) of ethanol for at least 2 hours. The ethanol was decanted, centrifuged at 14,000 RCF for 1 minute to remove particulate matter, and 1 ml of each clarified supernatant was transferred to an amber glass vial. Twenty microlitres of each sample was analysed via a Dionex (Sunnyvale, CA, USA), HPLC system equipped with a Polar Advantage II C18 monolithic column (3 µm, 4.6×50 mm, Dionex Sunnyvale, CA, USA) incubated in a thermostated (TCC100 40 °C) column oven and a UVD340U photo diode array detector. The mobile phase consisted of (A): water amended with 0.1% acetic acid, and (B): acetonitrile amended with 0.1% acetic acid. Binary gradient elution starting conditions were 35% B for 2 minutes, followed by an increase to 50% B at 4.5 minutes; 58% at 6 minutes; 70% at 7.29 minutes; 80% at 7.84 minutes; 95% at 8.5 minutes, holding for 0.5 minutes before returning to initial conditions. Commercially available verrucarin A (Sigma Chemical Co. St. Louis, MO, USA) was used as a standard, and absorbance at 260 nm was used to quantify this mycotoxin by the external standard method. The limit of detection in this system was 1 μg/ml, which corresponded to 16 μg per plate in this system. Crude samples of roridin D, E and H and verrucarin J provided by B. Jarvis (University of Maryland, USA) were used as qualitative standards. In the absence of reliable quantitative standards for these compounds, they were compared based on absorbance peak area. With this method, retention times for roridin D, verrucarin A, roridin E, verrucarin J, and roridin H were 1.9, 2.5, 5.5, 5.8, and 7.0 minutes, respectively.

### Experimental design and statistical analysis

The experiment was performed as a 2×9×4 (incubation conditions×media×incubation time), split-split design and completely, independently repeated three times. Values for sporulation were converted to $\log_{10}$ to normalise the error. Determination of the interactions and level of significance was analysed using proc mixed (SAS v. 9.1, Cary, NC, USA) and the pdmix800 macro (Saxton, 1998), which generated means, standard deviations and letter groupings of protected pairwise comparisons for each response variable (sporulation, specific toxin) for each time×light×medium interaction.

## 3. Results and discussion

The media used in this experiment are described in Table 1. Incubation time and media composition had statistically significant effects on the level of sporulation and accumulation of all macrocyclic trichothecenes. Additionally, the light/dark cycle and the two- and three-way interactions had statistically significant effects on sporulation and on most of the mycotoxin levels (Table 2). Sporulation was most strongly influenced by incubation time, but media type made the greatest contribution to trichothecene production.

The highest observed level of sporulation was seen on the high molasses media, (medium 3, Vogel's + 30 g/l of molasses) incubated in a light/dark cycle for 5 days, however statistically similar levels were also noted for several other combinations including PDA, high concentration of sucrose, and glucose (media 1, 7 and 5); in a light/dark cycle and in continuous darkness and at time points between 5 and 15 days (Table 2). While sporulation was generally higher with a light/dark cycle than without light, sporulation in the dark may be a commercially important finding because some mass productions systems cannot supply light. Earlier observations (Boyette, unpublished) were that sporulation in the dark did not occur with this strain of *M. verrucaria,* which would preclude some cost-effective methods of solid-state mass production. The present observations may have resulted from inadvertent genetic selection for an alternate phenotype during multiple serial transfers. Overall, light had a significant positive effect on sporulation, but the

**Table 2. Analysis of significant effects for sporulation and the accumulation of macrocyclic trichothecenes.**

| Effect [a] | Response variable | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sporulation | | Verrucarin A | | Roridin D | | Roridin E | | Roridin H | | Verrucarin J | |
| | F-value | Pr>F | F-value | Pr>F | F-value | Pr>F | F-value | Pr>F | F-value | Pr>F | F-value | Pr>F |
| Light/dark cycle (1) | 21.6 | ** | 192.9 | ** | 65.1 | ** | 3.1 | 0.081 | 2.0 | 0.158 | 1.6 | 0.2067 |
| Media (8) | 125.2 | ** | 296.2 | ** | 85.9 | ** | 45.5 | ** | 237.2 | ** | 140.4 | ** |
| Incubation time (3) | 314.4 | ** | 67.87 | ** | 29.9 | ** | 16.0 | ** | 122.2 | ** | 61.0 | ** |
| Light × Media (8) | 5.4 | ** | 26.5 | ** | 16.7 | ** | 1.4 | 0.186 | 3.1 | * | 3.1 | * |
| Light × Time (3) | 10.4 | ** | 15.1 | ** | 9.8 | ** | 3.8 | * | 4.2 | * | 1.42 | 0.2383 |
| Media × Time (24) | 25.5 | ** | 19.4 | ** | 11.3 | ** | 4.2 | ** | 26.4 | ** | 18.8 | ** |
| Light × Media × Time (24) | 3.3 | ** | 3.7 | ** | 2.9 | ** | 1.5 | 0.0798 | 2.1 | * | 0.9 | 0.6069 |
| Replication (2) | 0.2 | 0.086 | 1.8 | 0.1648 | 0.9 | 0.4196 | 3.3 | * | 0.6 | 0.5742 | 0.5 | 0.6371 |

[a] Values in parentheses are the degrees of freedom.
* Pr>F is <0.058.
** Pr>F is <0.0001.

effect was small. In contrast, there was a readily discernible role played by media composition. Medium 8, a defined, sucrose and ammonium sulphate-based medium, supported especially low levels of sporulation. After 5 and 9 days of incubation medium 8 had less than a tenth as many spores per plate as medium 6 and about one hundredth as many spores as medium 5. Because media 6 and 5 differed from medium 8 only in nitrogen source, it can be concluded that this concentration of nitrogen, in this form, is a poor choice for spore production. By comparing medium 6 with medium 7 (low and high sucrose media, respectively), and medium 4 with medium 3 (low and high molasses media, respectively), the effect of additional sugar is discernable. The higher sugar media supported 2- to 5-fold higher sporulation at most time points. In almost every combination of incubation time and light, the higher sugar media resulted in significantly higher sporulation.

It is standard practice to grow *M. verrucaria* on PDA for 5 days (Boyette *et al.*, 2008b) to 21 days (Anderson and Hallett, 2004), but the spore yield as a function of incubation time has not been reported. On PDA, the spore yield continued to increase through the entire 15-day observation period, under either dark or light incubation conditions (Table 3). Medium 8 also resulted in an overall increase in spore yield through day 15, but maximal spore yields occurred at earlier time points in other media. Factors that decrease the time for spore production may have great economic value in some production systems.

Media composition was the factor with the greatest effect on the accumulation of all detected trichothecenes (Table 2). Significant interactions with incubation time and the effect of light confound a simple description of the role of media, but many of the highest observed trichothecene levels were found with media 1 (PDA), 3 (high level of molasses), and 7 (high level of sucrose). These same substrates, with certain combinations of light and incubation time, sometimes yielded trichothecene levels that were not significantly different from zero, but more typically had the greatest trichothecene levels. Medium 8, especially in dark incubation, had notably high levels of verrucarin A (Table 4). This observation is unique because there was so little sporulation observed with this medium. In contrast, media 2, 5, 6 and 9 yielded levels of all measured trichothecenes that were generally below the limit of detection or within one standard deviation of zero (Table 4 and 5). Figure 1 includes two chromatographs to give an indication of the relative amount of trichothecenes present. The published extinction coefficients for verrucarin A, verrucarin J, roridin D, roridin E and roridin H are 17.7, 14.5, 21.4, 19.9 and 18.2, respectively (Cole and Cox, 1981). Correcting for the differences in the extinction coefficients, the ratio of verrucarin A:verrucarin J:roridin D:roridin E:roridin H was 1:9:0.75:0.23:4 for *M. verrucaria* grown 9 days in the light/dark cycle on PDA. Overall, all trichothecene levels were higher in continuous dark than in light/dark incubation (Table 4 and 5), but there were significant exceptions to that generalisation, and even in continuous darkness there were several media and time point combinations that had trichothecene levels not significantly different from zero. The levels of verrucarin J, roridin E and roridin H generally continued to increase with longer incubation time, up to and including 15 days. No clear pattern over time was observable in the accumulation of other trichothecenes.

**Table 3. Effect of incubation condition, media and time on the sporulation of *M. verrucaria*.**

| | Sporulation($\log_{10}$)[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Light/dark incubation | | | | Dark incubation | | | |
| Media[b] | 3 days | 5 days | 9 days | 15 days | 3 days | 5 days | 9 days | 15 days |
| 1 | 7.71 | 8.35 | 8.40 | 8.59 | 7.58 | 8.31 | 8.33 | 8.44 |
| 2 | 7.93 | 8.41 | 8.05 | 7.86 | 7.14 | 8.19 | 7.71 | 7.11 |
| 3 | 7.50 | 8.77 | 8.57 | 8.73 | 7.27 | 8.63 | 8.55 | 8.75 |
| 4 | 7.62 | 8.05 | 8.05 | 7.78 | 7.18 | 8.10 | 7.98 | 6.70 |
| 5 | 7.17 | 8.46 | 8.41 | 8.46 | 7.03 | 8.33 | 8.30 | 8.21 |
| 6 | 6.22 | 8.27 | 8.00 | 7.95 | 6.64 | 8.24 | 8.26 | 7.09 |
| 7 | 6.74 | 8.32 | 8.33 | 8.33 | 6.78 | 8.45 | 8.52 | 8.55 |
| 8 | 6.63 | 5.66 | 6.69 | 7.83 | 6.54 | 6.29 | 6.50 | 6.89 |
| 9 | 5.93 | 8.29 | 8.03 | 8.32 | 6.29 | 8.03 | 8.19 | 8.33 |

[a] Sporulation quantified by harvesting plates in water and measurement of colony-forming units after serial dilution. Results expressed as $\log_{10}$ conidia per 60 mm diameter Petri dish. Pooled standard error = 0.123. Average significant difference = 0.354.
[b] Complete media description given in Table 1.

**Table 4. Effect of incubation condition, media and time on the production of verrucarin A by *M. verrucaria*.**

| | Verrucarin A [a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Light/dark incubation | | | | Dark incubation | | | |
| Media [b] | 3 days | 5 days | 9 days | 15 days | 3 days | 5 days | 9 days | 15 days |
| 1 | *40* [c] | *56* | 540 | 268 | 231 | 370 | 540 | 501 |
| 2 | *165* | *94* | *63* | *65* | 232 | *173* | *150* | *114* |
| 3 | 492 | 1339 | 1238 | 1374 | 298 | 2134 | 1777 | 1876 |
| 4 | *166* | 179 | 105 | 123 | 201 | *173* | *150* | *127* |
| 5 | *1* | *50* | *4* | *2* | *13* | *147* | *65* | *30* |
| 6 | *22* | *33* | *7* | *25* | *28* | *75* | *17* | *3* |
| 7 | *72* | 371 | *117* | 375 | *34* | 964 | 908 | 1037 |
| 8 | 390 | 552 | 328 | 451 | 664 | 1310 | 1254 | 1428 |
| 9 | *46* | *141* | *92* | *54* | *40* | 318 | 263 | *124* |

[a] Verrucarin A combined accumulation, expressed as micrograms per 60 mm diameter Petri dish. Pooled standard error = 70.7. Average significant difference =197.6.

[b] Complete media description given in Table 1.

[c] Values in italics are within one significant difference of zero verrucarin A.

Complex relationships between fungal development and secondary metabolism are emerging in other systems (e.g. Calvo *et al*., 2002), however trichothecene biosynthesis by *M. verrucaria* is very poorly understood. A production system that minimised trichothecene levels while also yielding low spore concentrations would have limited applied value. Conversely, the need for worker safety may preclude use of a system with high spore production but was accompanied by unsafe levels of trichothecenes. Our experiments have tested only a few cultural conditions, but we have demonstrated here that sporulation and the biosynthesis of macrocyclic trichothecenes can be decoupled. For example, by dividing the number of spores produced in a given set of incubation conditions by the amount of verrucarin A, a ratio of spores per microgram of toxin can be obtained. In this study a range of conidia per microgram of verrucarin A was observed from $<10^6$ to $>10^9$ conidia (data not shown). By repeating this for other trichothecenes a pattern emerges where media 2, 4, 5 and 6 generally had the highest spore:trichothecene ratios and media 3, 7 and 8 had the lowest spore:trichothecene ratios. This pattern generally reflects the C:N ratio, where the lower C:N substrates more commonly produced higher spore:trichothecene ratios. Additional cultural conditions, such as temperature and pH, or other carbon and nitrogen sources may also contribute to maximising conidiation while minimising trichothecene levels. In the production of *M. verrucaria* separation of spores from the substrate would likely also remove substantial amounts of trichothecenes, but the growth media was included in the analysis in the present study to better understand conditions leading to trichothecene biosynthesis.

In addition to high spore yield with low trichothecene production, it is essential that the end product retains bioherbicidal activity. Macrocyclic trichothecenes are potent mammalian toxins (Abbas *et al*., 2002; Jarvis, 1991), therefore it would be desirable to identify a means of producing a bioherbicidally active formulation of *M. verrucaria* that was free of these products. Verrucarins and roridins, however, are known phytotoxins and have been implicated as virulence factors for *Myrothecium* spp. (Healey *et al*., 1994; El-Kassas *et al*., 2005). Trichothecene non-producing mutants of *F. graminearum* were less virulent but still pathogenic on wheat and maize (Proctor *et al*., 2002). A mycelial formulation of *M. verrucaria* with undetectable levels of verrucarin A was recently demonstrated to be bioherbicidally active (Boyette *et al*., 2008b). Ongoing research is also characterising the hydrolytic enzyme activities of this fungus and their role in pathogenicity (Hoagland *et al*., 2007).

**Table 5. Effect of incubation condition, media and time on the production of roridin H, verrucarin J, roridin E and roridin D by *M. verrucaria*.**

| Incubation condition | | | Trichothecene levels [a] | | | |
|---|---|---|---|---|---|---|
| Media [b] | Incubation time (days) | Light/dark cycle | Verrucarin J | Roridin H | Roridin E | Roridin D |
| 1 | 3 | light/dark | 8.1 | *7.1* | *1.9* | *1.4* |
| 1 | 3 | dark | 4.8 | *5.9* | *1.8* | *0.7* |
| 1 | 5 | light/dark | 18.7 | *8.9* | *2.1* | 2.8 |
| 1 | 5 | dark | 39.4 | 26.9 | 5.7 | 9.2 |
| 1 | 9 | light/dark | 69.8 | 39.5 | 2.4 | 8.4 |
| 1 | 9 | dark | 74.5 | 41.7 | 7.4 | 16.5 |
| 1 | 15 | light/dark | 61 | 40 | 6.7 | 4.7 |
| 1 | 15 | dark | 83.8 | 38.6 | 5.2 | 16.3 |
| 2 | 3 | light/dark | *0* | *0.8* | *0.1* | *0* |
| 2 | 3 | dark | *0* | *0.9* | *0* | *0* |
| 2 | 5 | light/dark | *0* | *0.1* | *0* | *0* |
| 2 | 5 | dark | *0.1* | *0.5* | *0* | *0* |
| 2 | 9 | light/dark | *0* | *0* | *0* | *0.7* |
| 2 | 9 | dark | *0.1* | *0.5* | *0* | *0* |
| 2 | 15 | light/dark | *0* | *0.1* | *0* | *0* |
| 2 | 15 | dark | *0.1* | *0.8* | *0.2* | *0* |
| 3 | 3 | light/dark | 6.4 | *4.9* | *1.6* | *0.9* |
| 3 | 3 | dark | *0.8* | *1.4* | *0.6* | *0* |
| 3 | 5 | light/dark | 29.4 | 19.3 | *2.1* | 2.3 |
| 3 | 5 | dark | 17.9 | 17.3 | 3.1 | 5 |
| 3 | 9 | light/dark | 28.9 | 26 | 2.5 | *0.9* |
| 3 | 9 | dark | 21 | 18.7 | *2* | 4 |
| 3 | 15 | light/dark | 28.8 | 23.9 | *2.4* | *0.3* |
| 3 | 15 | dark | 29.5 | 23.1 | 4.4 | 3.1 |
| 4 | 3 | light/dark | *0.4* | *0.9* | *0.1* | *0.1* |
| 4 | 3 | dark | *0* | *0.8* | *0.2* | *0* |
| 4 | 5 | light/dark | *0* | *0.3* | *0* | *0* |
| 4 | 5 | dark | *0* | *0.3* | *0* | *0* |
| 4 | 9 | light/dark | *0* | *0.2* | *0* | *0* |
| 4 | 9 | dark | *0.1* | *0.3* | *0* | *0* |
| 4 | 15 | light/dark | *0* | *0.1* | *0* | *0* |
| 4 | 15 | dark | *0* | *0.5* | *0* | *0* |
| 5 | 3 | light/dark | *1.6* | *1* | *0.8* | *0.2* |
| 5 | 3 | dark | *0* | *0.7* | *0* | *0* |
| 5 | 5 | light/dark | *1* | *2* | *0* | *1.5* |
| 5 | 5 | dark | *2* | *2.5* | *0* | *2* |
| 5 | 9 | light/dark | *2.1* | *2.1* | *0.8* | *0.4* |
| 5 | 9 | dark | *1.5* | *2.6* | *0.3* | *1.1* |
| 5 | 15 | light/dark | *2.5* | *2.8* | *0.6* | *0.3* |
| 5 | 15 | dark | *2.5* | *2.6* | *0.1* | *0.4* |
| 6 | 3 | light/dark | *1.1* | *0.7* | *1* | *0* |
| 6 | 3 | dark | *0* | *0.6* | *0* | *0* |
| 6 | 5 | light/dark | *0* | *0.3* | *0* | *0* |
| 6 | 5 | dark | *1.1* | *1.5* | *0* | *0.7* |
| 6 | 9 | light/dark | *1.1* | *1* | *0.5* | *0* |
| 6 | 9 | dark | *1.7* | *1* | *0.1* | *0.3* |
| 6 | 15 | light/dark | *1.2* | *0.7* | *0.5* | *0* |
| 6 | 15 | dark | *1.7* | *1.7* | *0.1* | *0* |

**Table 5. Continued.**

| Incubation condition | | | Trichothecene levels [a] | | | |
|---|---|---|---|---|---|---|
| **Media [b]** | **Incubation time (days)** | **Light/dark cycle** | **Verrucarin J** | **Roridin H** | **Roridin E** | **Roridin D** |
| 7 | 3 | light/dark | *2.3* | *0.9* | *1.7* | *0* |
| 7 | 3 | dark | *0* | *0.6* | *0* | *0* |
| 7 | 5 | light/dark | 23.7 | 13.8 | 5.1 | 2 |
| 7 | 5 | dark | 29.1 | 19 | 6.6 | 5.8 |
| 7 | 9 | light/dark | 20.3 | 10.5 | 3.4 | 0.5 |
| 7 | 9 | dark | 24.4 | 17.5 | 8.7 | 4.8 |
| 7 | 15 | light/dark | 30.8 | 20.5 | 9.8 | *1.5* |
| 7 | 15 | dark | 24.6 | 18.3 | 8.7 | 4.5 |
| 8 | 3 | light/dark | *0.9* | *0.6* | *0* | *0* |
| 8 | 3 | dark | *0.9* | *0.7* | *0.5* | *0* |
| 8 | 5 | light/dark | *1* | *3.4* | *0* | *0* |
| 8 | 5 | dark | *2.7* | *2.8* | *0* | *0.3* |
| 8 | 9 | light/dark | *0.8* | *1.1* | *0* | *0* |
| 8 | 9 | dark | *4* | *2.6* | *0* | *0.2* |
| 8 | 15 | light/dark | *0.5* | *3.6* | *0* | *0* |
| 8 | 15 | dark | *2.8* | *3* | *0* | *0.3* |
| 9 | 3 | light/dark | *1.3* | *1.3* | *0.5* | *0.1* |
| 9 | 3 | dark | *0* | *0.2* | *0* | *0* |
| 9 | 5 | light/dark | 8.4 | *6.7* | *0.9* | *1.2* |
| 9 | 5 | dark | 11.5 | *9.2* | 3.3 | 2.9 |
| 9 | 9 | light/dark | 8.8 | *8.6* | *1.9* | *1* |
| 9 | 9 | dark | 13.1 | *9.4* | 2.7 | 2.5 |
| 9 | 15 | light/dark | 8.9 | 13.1 | 3.3 | *0.5* |
| 9 | 15 | dark | 13.2 | 12.4 | 4 | *0.9* |
| | Pooled standard error | | 3.6 | 1.7 | 0.9 | 0.7 |
| | Average significant difference | | 10.1 | 4.7 | 2.4 | 2.0 |

[a] Mean trichothecene accumulation from three experiments, expressed as HPLC peak area.
[b] Complete media description given in Table 1.
[c] Values in italics are within one significant difference of zero peak area.

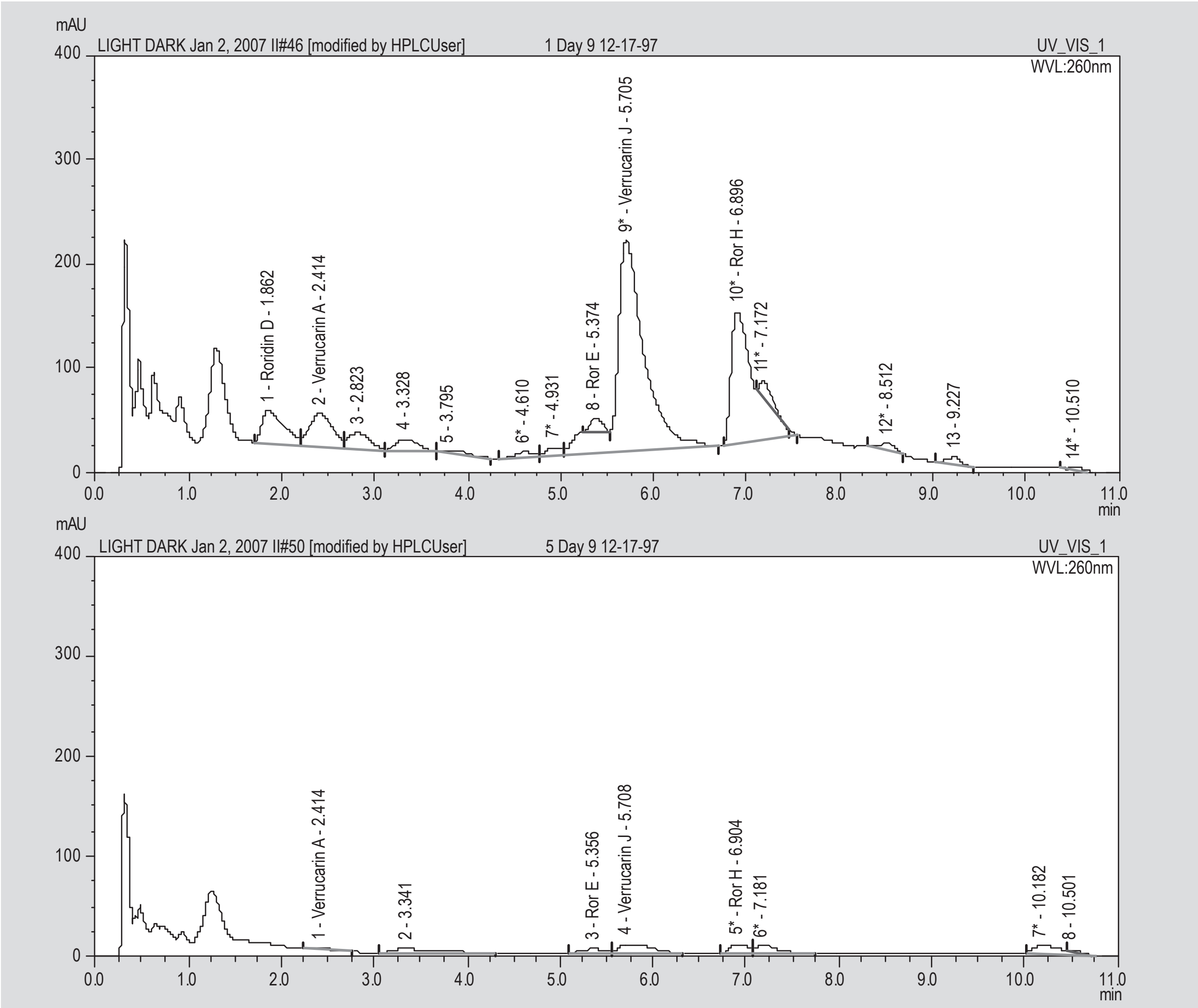


**Figure 1. Representative chromatographs of trichothecene extracts from *M. verrucaria* grown on different media. Top panel is from *M. verrucaria* grown for 9 days under 12 hour light-dark cycles on PDA. The lower panel is from the same conditions, but with a defined, glucose-based media (media 5, see Table 1).**

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## References

Abbas, H.K., Johnson, B.B., Shier, W.T., Tak, H., Jarvis, B.B. and Boyette, C.D., 2002. Phytotoxicity and mammalian cytotoxicity of macrocyclic trichothecene from *Myrothecium verrucaria*. Phytochemistry 59: 309-313.

Abbas, H.K., Tak, H., Boyette, C.D., Shier, W.T. and Jarvis, B.B., 2001. Macrocyclic trichothecenes are undetectable in kudzu (*Pueraria montana*) plants treated with a high-producing isolate of *Myrothecium verrucaria*. Phytochemistry 58: 269-276.

Anderson, K.I. and Hallett, S.G., 2004. Herbicidal spectrum and activity of *Myrothecium verrucaria*. Weed Science 52: 623-627.

Bluhm, B.H. and Woloshuk, C.P., 2005. Amylopectin induces fumonisin $B_1$ production by *Fusarium verticillioides* during colonization of maize kernels. Molecular Plant-Microbe Interactions 18: 1333-1339.

Boyette, C.D., Reddy, K.N. and Hoagland, R.E., 2006. Glyphosate and bioherbicide interaction for controlling kudzu (*Pueraria lobata*), redvine (*Brunnichia ovata*), and trumpetcreeper (*Campsis radicans*). Biocontrol Science and Technology 16: 1067-1076.

Boyette, C.D., Hoagland, R.E. and Weaver, M.A., 2008a. Interaction of a bioherbicide and glyphosate for controlling hemp sesbania in glyphosate - resistant soybean. Weed Biology and Management 8: 18-24.

Boyette, C.D., Weaver, M.A., Hoagland, R.E. and Stetina, K.C., 2008b. Submerged culture of a mycelial formulation of a bioherbicidal strain of *Myrothecium verrucaria* with mitigated mycotoxin production. World Journal of Microbiology and Biotechnology 24: 2721-2726.

Calvo, A.M., Wilson, R.A., Bok, J.W. and Keller, N.P., 2002. Relationship between secondary metabolism and fungal development. Microbiology and Molecular Biology Reviews, 66: 447-459.

Chang, P.-K., Bennett, J.W. and Cotty, P.J., 2002. Association of aflatoxin biosynthesis and sclerotial development in *Aspergillus parasiticus*. Mycopathologia 153: 41-48.

Cole, R.J. and Cox, R.H., 1981 Handbook of toxic fungal metabolites. Academic Press Inc., New York, NJ, USA.

El-Kassas, R., El-Din, Z.K., Beale, M.H. and Ward, J.L., 2005. Bioassay-led isolation of *Myrothecium verrucaria* and verrucarin A as germination inhibitors of *Orobanche crenata*. Weed Research 45: 212-219.

Etcheverry, M., Torres, A., Ramirez, M.L., Chulze, S. and Magan, N., 2002. *In vitro* control of growth and fumonisin production by *Fusarium verticillioides* and *F. proliferatum* using antioxidants under different water availability and temperature regimes. Journal of Applied Microbiology 92: 624-632.

Healey, P., Ng, T.J. and Hammerschlag, F.A., 1994. Response of leaf spot-sensitive and tolerant muskmelon (*Cucumis melo* L.) cells to the phytotoxin roridin E. Plant Science 97: 15-21.

Hestbjerg, H., Nielsen, K.F., Thrane, U. and Elmholt, S., 2002. Production of trichothecenes and other secondary metabolites by *Fusarium culmorum* and *Fusarium equiseti* on common laboratory media and a soil organic matter agar: an ecological interpretation. Journal of Agricultural and Food Chemistry 50: 7593-7599.

Hicks J.K., Yu J.-H., Keller N.P. and Adams T.H., 1997. *Aspergillus* sporulation and mycotoxin production both require inactivation of the FadA Gα protein-dependent signaling pathway. EMBO Journal 16: 4916-4923.

Hinojoa, M.J., Medina, A., Valle-Algarrab, F.M., Gimeno-Adelantadob, J.V., Jimeneza, M. and Mateo, R., 2006. Fumonisin production in rice cultures of *Fusarium verticillioides* under different incubation conditions using an optimized analytical method. Food Microbiology 23: 119-127.

Hoagland, R.E., Boyette, C.D., Weaver, M.A. and Abbas, H.K., 2007. Bioherbicides: research and risks. Toxin Reviews 16: 1-30.

Jarvis, B.B. 1991. Macrocyclic trichothecenes. In: Sharma, R.P. and Salunkhe, D.K. (eds.) Mycotoxins and phyoalexins in human and animal health. CRC, Boca Raton, FL, USA, pp. 361-421.

Klich, M.A., 2007 Environmental and developmental factors influencing aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus*. Mycoscience 48:71-80.

Llorens, A., Mateob, R., Hinojoa, M.J., Valle-Algarrab, F.M. and Jiménez, M., 2004. Influence of environmental factors on the biosynthesis of type B trichothecenes by isolates of *Fusarium* spp. from Spanish crops. International Journal of Food Microbiology 94: 43-54.

McCormick, S.P. and Alexander N.J., 2007. *Myrothecium roridum Tri 4* encodes a multifunctional oxygenase required for three oxygenation steps. Canadian Journal of Microbiology 53: 572-579.

Millhollon, R.W., Berner, D.K., Paxson, L.K., Jarvis, B.B. and Bean, G.W., 2003. *Myrothecium verrucaria* for control of annual morningglories in sugarcane. Weed Technology 17: 276-283.

Proctor, R.H., Desjardins, A.E., McCormick, S.P., Plattner, R.D., Alexander, N.J. and Brown D.W., 2002. Genetic analysis of the role of trichothecene and fumonisin mycotoxins in the virulence of Fusarium. European Journal of Plant Pathology 108: 691-698.

Reddy, K.V., Kumari, D.R. and Reddy, S.M., 1998. Effect of carbon and nitrogen sources on the interaction of mycotoxigenic fungi and mycotoxin production. Journal of Food Science 35: 268-270.

Saxton, A.M. 1998. A macro for converting mean separation output to letter groupings in Proc Mixed. In: Proceedings of the 23rd SAS User Group International Conference. SAS Institute, Inc., Cary, NC, USA, pp. 1243-1246.

Scheidegger, K.A. and Payne, G.A. 2003. Unlocking the secrets behind secondary metabolism: a review of *Aspergillus flavus* from pathogenicity to functional genomics. Journal of Toxicology - Toxin Reviews 22: 423-459.

Shim, W.-B. and Woloshuk, C.P., 1999. Nitrogen repression of fumonisin $B_1$ biosynthesis in *Gibberella fujikuroi.* FEMS Microbiology Letters 177: 109-116.

Trapp, S.C., Hohn, T.M., McCormick, S.P. and Jarvis, B.B., 1998. Characterization of the gene cluster for biosynthesis of macrocyclic trichothecenes in *Myrothecium roridum*. Molecular and General Genetics 257: 421-432.

Vogel, H.J., 1956. A convenient growth medium for *Neurospora* (Medium N). Microbiological Genetics Bulletin 13: 42-43.

Walker, H.L. and Tilley, A.M., 1997. Evaluation of an isolate of *Myrothecium verrucaria* from sicklepod (*Senna obtusifolia*) as a potential mycoherbicide agent. Biological Control 10: 104-112.

Weaver, M.A. and Lyn, M.E., 2007. Compatibility of a biological control agent with herbicides for control of invasive plant species. Natural Areas Journal 27: 264-268.

Yang, S. and Jong, S.C., 1995. Host range determination of *Myrothecium verrucaria* isolated from leafy spurge. Plant Disease 79: 994-997.